"Green synthesis of silver nanoparticles using *Ocimum sanctum* leaf extract and its anti-cancer activity against breast cancer cell line."

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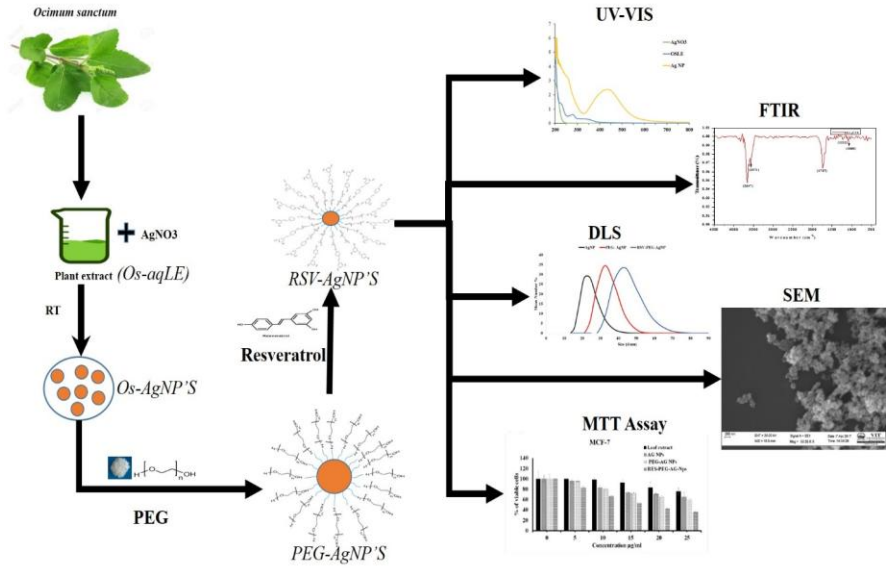
ABSTRACT ***Background:*** *Green synthesis of nanoparticles is a cost effective and eco-friendly, inspiring researcher towards biosynthesis of nanoparticles as superior over chemical and physical methods. The present study was designed for green synthesis of silver nanoparticles (AgNP's) using Ocimum sanctum leaf extract as reducing and stabilizing agent and its application in biological activities* ***Methods:****Ocimum sanctum leaf extract was used for the bio-reduction of AgNO3 to silver and then further characterized by UV-Visible Spectroscopy,Dynamic Light Scattering (DLS) Analysis, Fourier transform infra-red spectroscopy (FT-IR) and scanning electron microscope (SEM) techniques. SEM was used for determination of shape. Pegylation and Resveratrol was conjugated to AgNP'S followed by repeating the characterization. In vitro Antioxidant and antimicrobial was determined by using standard protocols. Cell viability was performed by MTT assay against breast cancer cell lines.* ***Results:*** *SEM analysis has confirmed synthesisedOs-AgNPs as spherical in shape. FTIR analysis revealed the possible involvement of phyto-constituents in both silver of leaf extract. DLS analysis exhibited the average hydrodynamic size of Os-AgNPs as 24.3nm.Os-AgNPs were found to be effective against pathogenic fungi and bacterial strains in comparison to the Ocimum sanctum leafextract. Meanwhile Os-AgNPs also showed an enhanced antioxidant properties than Ocimum sanctum leaf extract alone. Further Os-AgNPs were functionalised using PEG 8000 with a natural anticancer drug Resveratrol resulting RSV-AgNP'S showing significant reduction when compare to native Resveratrol. This comparative study of native resveratrol and PEGylated resveratrol on breast cancer cell lines (MDA-MB-231 and MCF-7) has revealed the application of nanoparticles in drug delivery systems.* ***Conclusion:****Current study states that O.sanctum leaves as a promising reducing agent for bio-reduction of AgNO3 (Ag^+ to Ag^0).Results have exhibited enhanced biological activities through which we can conclude green synthesis as a best and convenient method.*

Keywords: *Silver nanoparticles, PEG, Ocimum sanctum, anti-bacterial, anti-cancer activity.*

Graphical Abstract:



Introduction:

Biological synthesis of Nanoparticles is an emerging trend due to its nontoxic effect and eco-friendly by-products. Metal nanoparticles have several properties which includes catalytic activity, optical property, electronic property, anti-bacterial property, and magnetic property (1-4). Biomolecules present in plants

like alkaloids, flavonoids, saponins, steroids, tannins has the ability to act as both reducing and capping agents (5). Studies have reported that the process of green synthesis is always extracellular and very short over microbial synthesis (6, 7).

Silver nanoparticles (Ag NPs) have drawn tremendous attention in recent days due to its efficient applications areas in the field medical (8), electronic (9), catalytic (10) and optical applications(11). Various chemical and physical methods have been used for the synthesis of Ag NPs like chemical reduction(12, 13), electrochemical (14), irradiation (15, 16) and thermal decomposition (17), as well as the green chemistry route (18). In biological method bacteria (19) and fungus (20) were used in preparing Ag NPs, recently Plant based Ag NPs synthesis drawn much attention due to its simple, rapid, non-toxic, dependable, reproducible and can produce well-defined size under controlled conditions (21, 22).

Synthesized Ag NPs are stabilized to avoid aggregation using surface passivation agents like surfactant molecules and polymers. Polyethylene glycol (PEG) is well known stabilizer used in synthesis of Ag NPs (23) where its steric hindrance avoid aggregation of Ag NPs (24, 25). Surface modification/functionalization of nanoparticle is a crucial step in facilitating Ag NPs application to medical, biotechnology and translational research (26).

In this present study, we have used the leaf extract of *Ocimum sanctum* (*O. sanctum*) for green synthesis of AgNps. *O. sanctum* an aromatic, perennial plant commonly known as "holy basil" belongs to laminaceae family. This plant is well known for its medicinal use and in Ayurveda for its various healing properties from ancient times (27, 28) and used for the treatment of bronchitis, malaria, diarrhea, dysentery, skin diseases, arthritis, painful eye diseases, chronic fever etc. (29). It shows anticancer, antidiabetic, antimicrobial, hepatoprotective, cardioprotective, analgesic and diaphoretic actions etc.,(30-32). Major active constituents of tulasi includes, carvocrolursolic acid, rosmarinic acid, eugenol, oleic acid etc.(33-35).

In this study, we adopted green synthesis of Ag NPs using leaf extract of O. sanctum using optimized titration and temperature method. The surface of synthesized Ag NPs was stabilized using PEG 8000 and then factionalized by conjugating with standard neoplastic drug RESVERATROL for biological applications. The synthesized *AgNps* and drug Conjugated *RSV-AgNP'S* were characterized using UV–vis analysis, FTIR, DLS, SEM. Further their biological activity was assessed using anti-oxidant, anti-microbial and anti-cancer assays.

Materials and methods:

Silver nitrate (AgNO3), PEG (Mw 8000) and Resveratrol was purchased from (Sigma Aldrich chemicals, India). *Ocimum sanctum* leaves was collected from premises of Pondicherry University, India. All the solutions were prepared using double distilled water. The reagents used in all the experiments are of analytical grade and high purity.

Collection and Preparation of plant Extract:

Collected *O.sanctum* leaves were washed thoroughly with double distilled water. Aqueous extract was prepared by boiling 5g of leaves in 100 ml of deionized water at 30 ^{0}C for 30 minutes. The cooled solution was filtered by using Whatman Filter Paper No.1 and stored at 4 ^{0}C for synthesis of silver nanoparticles.

Green synthesis of silver nanoparticles and purification:

For the reduction of silver nitrate AgNO3, 10 ml of aqueous extract is added to 30ml of silver nitrate solution under continues agitation for 2 hours. The sample is left under room temperature for 12 hours where the colour change from pale yellow to yellowish brown was observed indicating formation of Ag NP's. Synthesized AgNP's was purified by repeated centrifugation with deionized water at 12000rpm for 20 mins. Purified AgNP's was dispersed in to deionized water for characterization.

Functionalization of *Os-AgNP's:*

Functionalization of *AgNP'S* were carried out using stabilizer polyethylene glycol (PEG) followed by conjugation with standard anti-cancer drug Resveratrol (RES). 3 mg/3ml of synthesized *AgNP's* solution was agitated continuously by adding 60 ml of 0.5% PEG 8000 with speed of 150rpm at 50 °C. Following Pegylation, conjugation was performed by adding three different concentrations (25ug ml^{-1},50ug ml^{-1} and 150 ug ml^{-1}) of resveratrol to 3ml of PEGylated solution each. The prepared solution is kept under continuous stirring overnight in dark place at room temperature. Further incubation is followed by purification by centrifuging the solution at 14000rpm for 1 hrs. The pellet is collected and vacuum dried for characterization analysis.

Characterization of *Os-AgNP's,PEG-AgNP'S*and *RSV-AgNP'S*:

U.V-Vis spectroscopy () analysis was performed to confirm the synthesis of *AgNP'S* synthesis by *O.sanctum* leaf extract, PEGylated*AgNP'S* and *RSV-AgNP'S* by their changes in absorption peak due to Surface Plasmon Resonance (SPR) at the range of 200-800nm.The hydrodynamic size of synthesized *Os-AgNP's, PEG-AgNP'S* and *RSV-AgNP'S* were studied by the measurements took at 25°C using Dynamic Light Scattering (DLS) analysis (). Morphological studies of *AgNP'S*, *PEG-AgNP'S* and *RSV-AgNP'S* was done by using **Scanning** Electron Microscope (JEOL 6360 TESCAN). The FTIR spectra of ***O.sanctum* leaf extract**,*AgNP's*, *PEG-AgNP'S* and *RSV-AgNP'S* were recorded using KBR as reference by (Thermo Nicolet nexus 6700) spectrometer ranging from 500-4000 cm^{-1} at a resolution 4 cm^{-1}.

Cell lines and culture conditions:

MCF-7, MBA-MB-231 and NIH 3T3 cells were obtained from NCCS, PUNE. Cells were grown in growth medium supplemented with 10% FBS and 1% antibiotics (penicillin–streptomycin) with 5% CO_2 and 37°c temp.

MTT assay:

Cytotoxic properties of synthesized AgNP's and *RSV-AgNP'S*was assessed by employing MTT (3-[4,5-dimethylthiazol-2-yl] 2, 5-diphenyltetrazolium bromide) dye reduction assay. NIH 3T3, MCF-7 and MBA-MB-231 cells (1 × 105/well) l were plated in 96 well plate. After 24hrs cell were treated with different concentrations of *AgNP's* and *RSV-AgNP'S* (25 µg/ml, 50 µg/ml and 100 µg/ml) for 24, 48 and 72 hrs. Following, contents in each well is carefully removed and then, MTT solution (5 mg/ml of MTT dissolved in PBS) was added and incubated for 3 hours. Then, formazan crystals were dissolved using DMSO for 30mins and absorbance was recorded at 570 nm in UV-VIS spectrophotometer. The % cell inhibition was determined using following formula.

$$\%\ \text{cell Inhibition} = \frac{\text{Abs (Control)} - \text{Abs (sample)}}{\text{Abs (control)}} \times 100$$

The Inhibitory concentration required for 50% cytotoxicity (IC50) value was determined.

Statistical analysis:

Statistical analysis was carried out using Prism statistical software package. All results were expressed in mean ± SD of three individual experiments, employing Student T-Test analysis. Values $p < 0.05$ was considered as significant.

3.0. Result and Discussion:

3.1. Synthesis of *Os-AgNps, PEG-AgNP'S* and *RSV-AgNP'S:*

In this present study we have used aqueous leaf extract of *O. sanctum (OS-aqLE)* as a reducing agent for the synthesis of AgNO3 nanoparticles. We have added 10ml of prepared aqueous leaf extract of *O. sanctum* to 30ml 1mM of AgNO3 solution at room temperature and incubated for 12hrs. The formation of *AgNps* were confirmed from yellow to yellowish brown due to the reduction of silver nitrate solution to silver nanoparticles(Figure 1A). This change in colour of solution occurs due to the Surface Plasmon Resonance excitation (SPR) of *AgNPs(36)*, the resulted SPR peak depends on the size and shape of nanoparticle(37). Synthesized nanoparticles were PEGylated with 0.5% of PEG-8000 by adding 20ml to 3ml of 3mg silver nanoparticle followed by the procedure above mentioned in methods. The resulted pellet of PEG-AgNP'S was further conjugated with an anticancer drug Resveratrol (RSV). The SPR peaks of resulted pellet after Pegylation (PEG-AgNP'S) and conjugation (RSV-AgNP'S) were analyzed with the help of UV-VIS spectroscopy.

3.3. Characterization of synthesized *Os-AgNP'S, PEG-AgNP'S* and *RSV-AgNP'S*:

3.3.1 U.V-Vis spectroscopy analysis:

U.V –Vis spectroscopy analysis were shown in figure 1. According to Mie theory, only a single SPR band is expected in the absorption spectra of spherical nanoparticles. The results of synthesized *Os-AgNP'S* has resulted a characteristic absorption peak at 450 nm confirming the synthesis of silver nanoparticles and the time dependent U.V Vis spectrum recorded was shown in figure 1B & 1C,indicating the stability of NPs in aqueous medium (Daizy Philip et al., 2011).The U.V-Vis spectra of *PEG-AgNP'S* was shown in figure1D,which reveals that the synthesized nanoparticles has chemical interaction causing lower electron conductivity in the outermost atomic layer, resulting the red-shifts (Mandal A. et al., 2012).Followed by the Pegylation, the conjugation of anti-cancer drug resveratrol to functionalized nanoparticles has shown in figure1Dwith shift and decrease in absorption intensity which attributes to decrease in distance between the particles due to the result of binding(38).

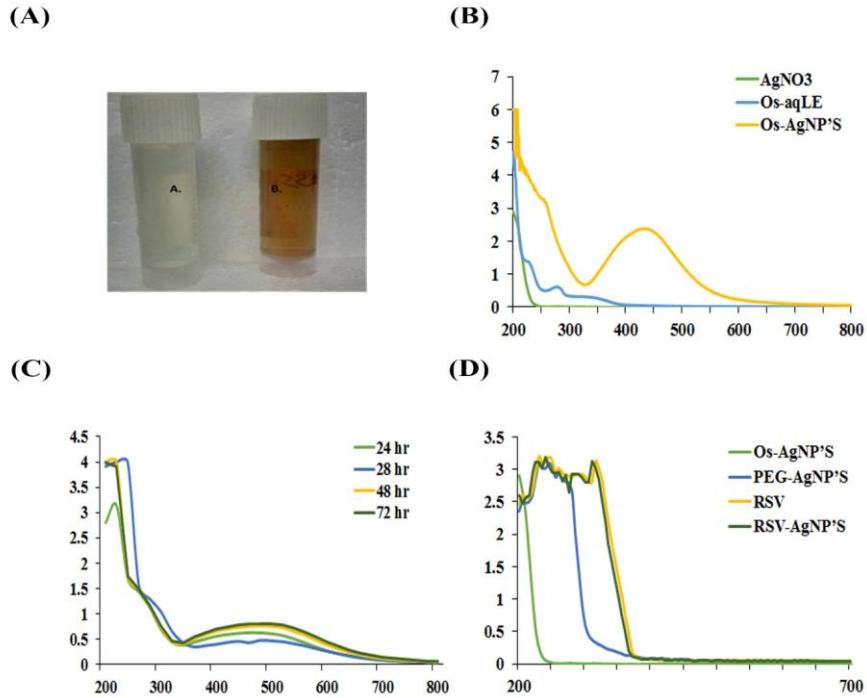


Figure 1: The colour change in reaction mixture due to SPR indicates AgNP'S synthesis (A),U.V-Vis spectroscopy of AgNO3,O.sanctum leaf extract, *Os-AgNP'S* (B),Time dependant analysis of *Os-AgNP'S* (C),Red shift of the SPR peak for *PEG-AgNP'S* and *RSV-AgNP'S.*

3.3.2 FTIR studies:

In present study, to analyze and identify the major functional groups responsible for formation, functionalization and conjugation of *AgNP's*, we have acquired the FTIR spectra of O. sanctum leaf extract, *Os-AgNP'S*, *PEG-AgNP'S* and *RSV-AgNP'S* shown in Figure 2.The IR spectra of *Os-AgNP'S* revealed the peaks related to functional group which are accountable to stretching vibrations of hydroxyl groups (3000–3300 cm−1), CH2 and CH3 functional groups (2800–3000 cm−1), C=C groups or C=O groups of aromatic compounds and carboxylic acids respectively (1626 cm−1), amide I (CONH2) and amide II (CONH) groups (1400–1550 cm−1), germinal methyl's (1380–1403 cm−1) and ether linkages and C–O or C–O–C functional groups (1000–1100 cm−1) in O. sanctum leaf extract spectra(39). The band at 1318 cm-1 attributed to–C–O stretching mode(40).The bands at 1634 and 1610cm-1 may result from the –C¼C– stretching vibration(41).The bands at 1017 and 1027cm-1 can be assigned as absorption peaks of–C–O–C(42). The IR spectra of *PEG-AgNP'S* has revealed the bands of C–O stretching mode which were merged in a very broad envelope centered on 1268 and 1007 cm−1 arising from C–O, C–O–C stretches and C–O–H bends vibrations of Ag NPs in PEG. Also, the aliphatic C–H stretching, in 1413 and 1344 cm−1 were due to C–H bending vibrations (Mandal A. et al., 2012). Absence of O. sanctum AgNPs characteristic peaks indicate the removal of those functional groups by repeated washes prior to PEGylation. The broad peaks in 503, 407 and 291 cm−1 are related to Ag NPs banding with oxygen from hydroxyl groups of PEG chains. Therefore, the FT-IR spectra showed the existence of van der Waals interactions between the chain of PEG and AgNPs in the polymeric media (Karakotia et al., 2012). The IR spectra of resveratrol was shown in Figure 2with the peaks at 3265 and 1604 cm−1 assigning to the hydroxyl stretching vibration and aromatic double-bond stretching vibration. The, C-O stretching, and C-C bending, C-H bending vibrations of the benzene ring in the fingerprint region around 1350– 1000 cm−1 confirms the process of drug conjugation.

3.3.3 DLS:

The size of the AgNPs were accessed by employing Dynamic light scattering method, where the size is measured by means of hydrodynamic radius(43). The average hydrodynamic size of *Os-AgNP'S, PEG-AgNP'S* and *RSV-AgNP'S* were shown in figure 2F.The result of DLS indicates that the size of silver nanoparticles were 24.3nm with Poly Dispersity Index (PDI) of 0.390 .whereas the size of Os-AgNP'S has increased to 32.6nm with PDI value 0.452 after Pegylation. Furthermore an increase in size to 43.8nm with PDI of 0.346 was observed upon conjugation with Resveratrol. An increase in size indicates successful conjugation of drug to silver nanoparticles and the PDI value < 1 indicates the particles are polydispersed without agglomeration.

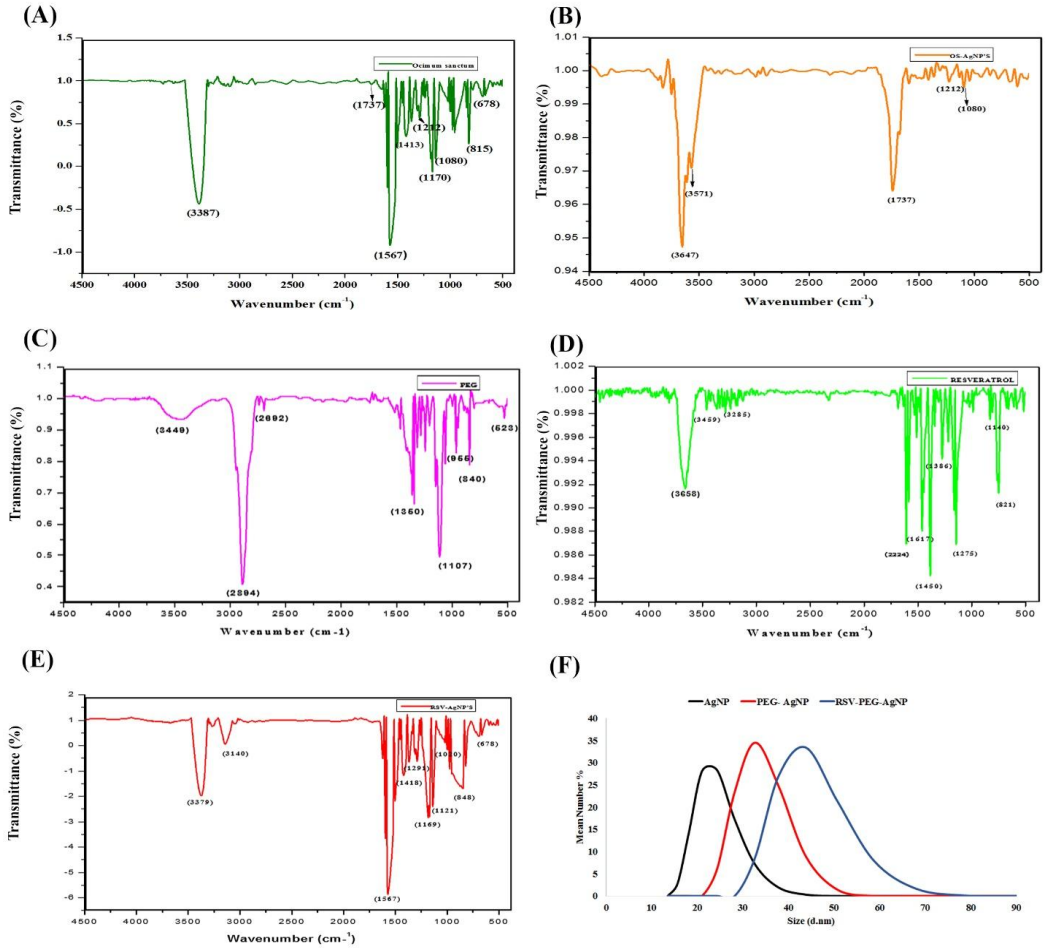


Figure 2: FTIR spectral analysis of O.sanctum leaf extract (A),Synthesised Os-AgNP'S indicating involvement of plant biological compounds during reduction (B), Spectra of PEG (C),Spectra of Resveratrol (D),Resveratrol conjugated Silver nanoparticles RSV-AgNP'S (E), DLS anlaysis showing an increase in size of AgNP'S after Pegylation (PEG-AgNP'S) and drug conjugation with Resveratrol (RSV-AgNP'S) (F).

3.3.4. SEM:

The morphological studies were analyzed by SEM.The SEM micrograph images of *Os-AgNP'S, PEG-AgNP'S* and *RSV-AgNP'S* were shown in Figure 3.The micrograph image in Figure 3A shows a uniform distribution of spherical*Os-AgNP'S*. The larger size of AgNPs is due to the aggregation of smaller NPs. In Figure 3B the *PEG-AgNP'S* were observed to be well separated without agglomeration due to surface coating of Poly Ethylene Glycol and diminishing the high surface energy resulting non-aggregated distinct particles (Karakotia et al., 2012). The morphology of drug conjugated nanoparticles observed in Figure 3C

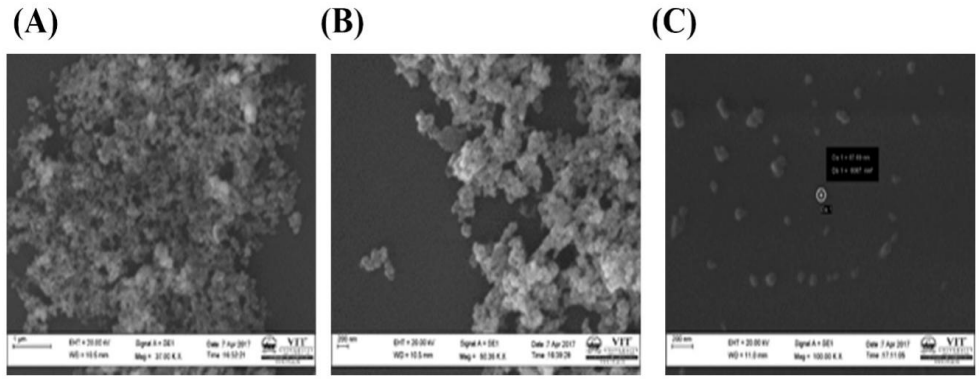


Figure 3: SEM analysis of Os-AgNP'S (A),PEG-AgNP'S (B), and RSV-AgNP'S.

3.6 Anti- cancer activity:

In-vitro cytotoxicity activity of *Os-aqLE*, *Os-AgNP'S, PEG-AgNP'S* and *RSV-AgNP'S* against non-cancerous (HEK-293) and cancerous cell lines (MCF-7 and MDA-MB-231 cells) were assessed using MTT colorimetric assay at 48h incubation. Results of MTT assay were showed in Figure 4. Untreated cells were considered as control. In agreement with other studies(44-46), our synthesized nanoparticles showed significant cytotoxicity activity compared to untreated population. *Os-aqLE* has shown minimal cytotoxic effect towards HEK-293 cells and as well as MCF-7 and MDA-MB-231 cells as *Os-AgNP'S, PEG-AgNP'S*and *RSV-AgNP'S*. Interestingly synthesized*Os-AgNP'S* has shown minimal cytotoxic effect against non-cancerous cell line HEK-293 cells line. However, the anti-cancer effect of *Os-AgNP'S* is high towards MCF-7 and MDA-MB-231 cells in a dose-dependent manner. Whereas *PEG-AgNP'S* has shown similar trend like *Os-AgNP'S.* Fascinatingly the cytotoxic effect of *RSV-AgNP'S* is significantly high in cancerous cells when compared to *Os-AgNP'S and PEG-AgNP'S*, indicating that resveratrol conjugated nanoparticles have increased the efficiency of nanoparticles to target cancer cell by slow release of drug inside the cell(47). The calculated IC50 values of *Os-AgNP'S* against MCF-7 and MDA-MB-231 is 32.86 µg/ml and 26.23µg/ml respectively and for *PEG-AgNP'S* 29.47µg/ml and 21.47 µg/ml against MCF-7 and MBA-MB-231 cells. Whereas the *RSV-AgNP'S* IC_{50} values are 17.60µg/ml and 15.39 µg/ml against to MCF-7 and MDA-MB-231 respectively. The plant extract alone has showed minimal cytotoxicity activity when compared to synthesized nanoparticles. The cytotoxicity of *Os-AgNP'S, PEG-AgNP'S* and *RSV-AgNP'S*is more towards MDA-MB-231cells than MCF-7 cells. This increase in cytotoxicity of synthesized Np's may be due to increase in cellular uptake and retention of NP's in cells(48).

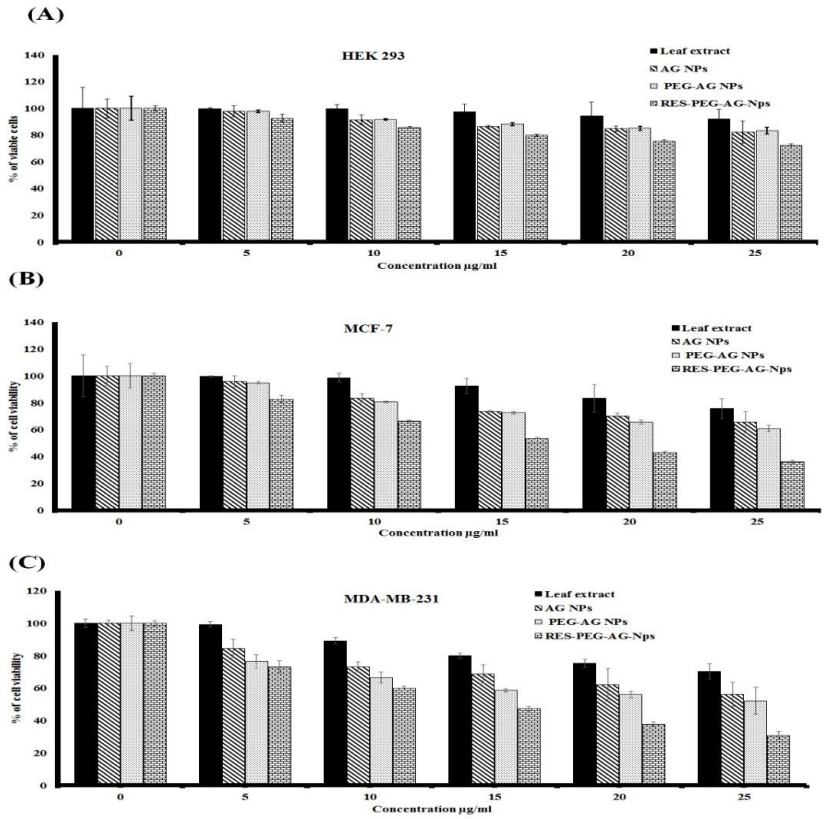


Figure 4: Dose dependant in-vitro Cytotoxicity assay of O.sanctum leaf extact, Os-AgNP'S, PEG-AgNP'S and RSV-AgNP'S on non-cancerous cell lines HEK-293 (A), breast cancer cell lines MCF-7 (B), and MDA-MB 231 (C).

Conclusion:

In conclusion, the present study shows promising reducing property of O.sanctum leavesbio-reduction of AgNO3 (Ag^+ to Ag^0). An eco-friendly and fast facile synthesis of *Os-AgNP'S* by O.sanctum leaf extract is established. Further surface modification is done to conjugate with standard drug resveratrol. Characterization studies were performed for synthesized, *Os-AgNP'S, PEG-AgNP'S* and *RSV-AgNP'S*. Our MTT assayrevealed significant anti-cancer effect of *RSV-AgNP'S*towards MCF-7 and MDA-MB-231 cells than *Os-*

*AgNP'S*and *PEG-AgNP'S*. In sum, our study have increased the anti-cancer efficiency of resveratrol with the help of AgNPs delivery into cells.

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Author contribution:
SSVand PL have designed the experiments. SSVhas performed the experiments. SSV and PL analyzed the data and drafted the manuscript.

Conflict of interest:
There is no conflict of interest reported by the authors.

Reference:

1. De Gaetano F, Ambrosio L, Raucci M, Marotta A, Catauro M. 2005. Journal of Materials Science: Materials in Medicine 16: 261-5
2. Crabtree JH, Burchette RJ, Siddiqi RA, Huen IT, Hadnott LL, Fishman A. 2003. Peritoneal Dialysis International 23: 368-74
3. Królikowska A, Kudelski A, Michota A, Bukowska J. 2003. Surface science 532: 227-32
4. Zhao G, Stevens SE. 1998. Biometals 11: 27-32
5. Madkour LH. 2018. Chronicles of Pharmaceutical Science 2: 384-444
6. Shankar SS, Rai A, Ahmad A, Sastry M. 2004. Journal of colloid and interface science 275: 496-502
7. Ramteke C, Chakrabarti T, Sarangi BK, Pandey R-A. 2012. Journal of chemistry 2013
8. Cao X, Cheng C, Ma Y, Zhao C. 2010. Journal of Materials Science: Materials in Medicine 21: 2861-8
9. Mohan YM, Lee K, Premkumar T, Geckeler KE. 2007. Polymer 48: 158-64
10. Feng X, Qi X, Li J, Yang L, Qiu M, et al. 2011. Applied Surface Science 257: 5571-5
11. Hayward R, Saville D, Aksay I. 2000. Nature 404: 56
12. Narayanan R, El-Sayed MA. 2005. Catalysis with transition metal nanoparticles in colloidal solution: nanoparticle shape dependence and stability. ACS Publications
13. Shameli K, Ahmad MB, Zargar M, Yunus WMZW, Ibrahim NA, et al. 2011. International journal of nanomedicine 6: 271
14. Eustis S, El-Sayed MA. 2006. Chemical society reviews 35: 209-17
15. Mansor A, Kamyar S, Majid D, Yunus WMZW, Nor AI, et al. 2009. Research Journal of Biological Sciences 4: 1156-61
16. Shameli K, Ahmad MB, Yunus WMZW, Rustaiyan A, Ibrahim NA, et al. 2010. International journal of nanomedicine 5: 875
17. Navaladian S, Viswanathan B, Viswanath R, Varadarajan T. 2007. Nanoscale research letters 2: 44
18. Sharma VK, Yngard RA, Lin Y. 2009. Advances in colloid and interface science 145: 83-96
19. Gou Y, Zhou R, Ye X, Gao S, Li X. 2015. Science and technology of advanced materials 16: 015004
20. Govindappa M, Farheen H, Chandrappa C, Rai RV, Raghavendra VB. 2016. Advances in Natural Sciences: Nanoscience and Nanotechnology 7: 035014
21. Saxena A, Tripathi R, Zafar F, Singh P. 2012. Materials letters 67: 91-4
22. Mittal AK, Chisti Y, Banerjee UC. 2013. Biotechnology advances 31: 346-56
23. Popa M, Pradell T, Crespo D, Calderón-Moreno JM. 2007. Colloids and Surfaces A: Physicochemical and Engineering Aspects 303: 184-90
24. Chen Z, Gao L. 2007. Materials Research Bulletin 42: 1657-61
25. Shkilnyy A, Soucé M, Dubois P, Warmont F, Saboungi M-L, Chourpa I. 2009. Analyst 134: 1868-72
26. Luo C, Zhang Y, Zeng X, Zeng Y, Wang Y. 2005. Journal of colloid and interface science 288: 444-8
27. Staples G, Kristiansen MS. 1999. Ethnic Culinary Herbs: A Guide to Identification and Cultivation in Hawai? i: University of Hawaii Press
28. Warrier P, Nambiar V, Ramankutty C, Vaidya SA. 1995.
29. Sundaram RS, Ramanathan M, Rajesh R, Satheesh B, Saravanan D. 2012. Journal of Liquid Chromatography & Related Technologies 35: 634-50
30. Prakash P, Gupta N. 2005. Indian journal of physiology and pharmacology 49: 125
31. Sethi J, Sood S, Seth S, Talwar A. 2004. Indian Journal of Clinical Biochemistry 19: 152-5
32. Rai Va, Iyer U, Mani U. 1997. Plant foods for human nutrition 50: 9-16
33. Chattopadhyay R, Sarkar S, Ganguly S, Medda C, Basu T. 1992.
34. Devi PU, Ganasoundari A, Rao BSS, Srinivasan KK. 1999. Radiation Research 151: 74-8
35. Anderson TJ, Meredith IT, Yeung AC, Frei B, Selwyn AP, Ganz P. 1995. New England Journal of Medicine 332: 488-93

36. Abadi Mn, Agarwal A, Barham P, Brevdo E, Chen Z, et al. 2016. arXiv preprint
37. Pal S, Tak YK, Song JM. 2007. Applied and environmental microbiology 73: 1712-20
38. Ghosh SK, Pal T. 2007. Chemical reviews 107: 4797-862
39. Socrates G. 2004. Infrared and Raman characteristic group frequencies: tables and charts: John Wiley & Sons
40. Trchová M, Šeděnková I, Tobolková E, Stejskal J. 2004. Polymer Degradation and Stability 86: 179-85
41. Katritzky AR, Ramsden CA, Joule JA, Zhdankin VV. 2010. Handbook of heterocyclic chemistry: Elsevier
42. Kahlenberg V, Wertl W, Többens D, Kaindl R, Schuster P, Schottenberger H. 2008. Zeitschrift für anorganische und allgemeine Chemie 634: 1166-72
43. Gallego-Urrea JA, Tuoriniemi J, Hassellöv M. 2011. TrAC Trends in Analytical Chemistry 30: 473-83
44. Krishnaraj C, Muthukumaran P, Ramachandran R, Balakumaran M, Kalaichelvan P. 2014. Biotechnology Reports 4: 42-9
45. Lalitha P. 2015. Progress in biomaterials 4: 113-21
46. Sivaraj R, Rahman PK, Rajiv P, Narendhran S, Venckatesh R. 2014. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 129: 255-8
47. Panyam J, Zhou W-Z, Prabha S, Sahoo SK, Labhasetwar V. 2002. The FASEB journal 16: 1217-26
48. Prabhu D, Arulvasu C, Babu G, Manikandan R, Srinivasan P. 2013. Process Biochemistry 48: 317-24

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49. Liu J, Lu Y (2006) Preparation of aptamer-linked gold nanoparticle purple aggregates for colorimetric sensing of analytes. Nat Protoc 1: 246-252.